Available Online at

http://www.ijcpa.in

October - December 2016

DOI : http://dx.doi.org/10.21276/ijcpa

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726 ; pISSN : 2395-2466

Research Article | Volume-4 | Issue-1 | Article ID: 1189

EVALUATION OF *IN VITRO* ANTICANCER POTENTIALS OF PVP STABILIZED SILVER, COPPER AND NICKEL NANOPARTICLES

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Received: 2 December 2016 / Revised: 10 December 2016 / Accepted: 12 December 2016 / Available online: 12 December 2016

ABSTRACT

Despite formidable global efforts, cancer continues to be the leading cause of human death. Prevailing anticancer drugs, although effective in controlling neoplastic growth, the eventual toxicity on normal cells and other health complications caused by them are inevitable. The metal nanoparticles by virtue of their specificity and selective toxicity are promising to be safe anticancer drugs. In this study, Polyvinyl Pyrrolidone (PVP) capped three transition metal nanoparticles namely copper (PVP-CuNP), nickel (PVP-NiNP) and silver (PVP-AgNP) were synthesized by bottom up wet chemical method. Using MTT assay the cytotoxic and anticancer potentials of these nanoparticles were tested against normal (Vero) and human lung cancer (A549) cells in vitro. The cytotoxicity and anticancer efficacy of these nanoparticles were determined in terms of IC_{50} value and longevity of inhibitory effect on relative growth rate (RGR). Continuous culturing of cells in the presence of IC_{50} doses of these nanoparticles was carried out to determine the longevity of their anticancer activity. The toxic doses (µg/mL) of PVP-CuNP, PVP-NiNP and PVP-AgNP on Vero (normal) cell lines respectively were 11.80, 6.70 and 6.35, while were 1.75, 1.90 and 1.40 on A549 cells. The longevity of anticancer efficacy in terms of displaying qualified grade of activity of these nanoparticles were 3, 5 and 3 days respectively. Overall, the silver nanoparticles (PVP-AgNP) demonstrated better efficacy and moderate cytotoxicity with longer anticancer activity at minimal doses.

Key words: *Metal nanoparticles, PVP, MTT assay, Cytotoxicity, Anticancer*

1. INTRODUCTION

Cancer, uncontrolled growth and spread of anomalistic cells leading to malfunctioning of healthy system, is considered as a major precarious disease threatening human kind. The burden on health care caused by cancer has been on the increasing trend during the recent decades primarily because of changing lifestyle, food habits, environmental pollution and risk behaviors amongst vulnerable populations. Globally 13% of annual deaths are cancer related and two third of these occur mainly in low and middle income countries[1]. Cancer has been a challenging disease particularly in developing countries as the percentage of new cancer cases has dramatically increased from 15% in 1970 to 56% in 2008 and it is estimated that by the year 2032 it can rise up to 70%[2].

Although the overall incidence of cancer in India is low compared to that of high-income countries[3], India still bears over a tenth of the global burden of cancers. Annually, approximately 1 million women and men are newly diagnosed with cancer and over 700,000 die as a result of malignancies[4]. The five most common cancers recognized among males are lung, head and neck, prostate, stomach and large bowel. In women, these are breast, cervix, ovary, oral and stomach cancer[5]. In major developing countries like China and India with massive populations the global contribution of cancer burden is high and they face local challenges to control and treat cancer[6].

Development of cancer in most cases is due to the occurrence of mutation in tumor suppressor genes and activation of proto-oncogenes. This causes genetic alterations resulting in differentiation and neoplastic growth of cells. Chemotherapy is one of the methods of treating cancer through administration of antineoplastic or anticancer drugs aiming at eradicating the neoplastic cells or reducing the number of cells. The anticancer drugs are categorized as alkylating agents (causing direct damage of DNA), antimetabolites (interfering with synthesis of DNA and RNA) and antitumor antibiotics (interfere with enzymes required for DNA replication). The anticancer drugs, although effective at the initial stages of cancer development, their efficacy wans with advanced stages. This is because of the genetic alterations occurring in tumor cells which make them behave unpredictably and differently respond to the drugs leading to alterations in treatment sensitivity. Therefore, the anticancer drugs could not exert their complete efficacy uniformly with all tumor cells and fail in many cases[7].

Research studies conducted in recent years report certain contraindications associated with anticancer drugs. As the anticancer drugs lack selective specificity for neoplastic cells they tend to eliminate normal healthy cells as well, especially those divide often e.g., immune cells. Thus, the organ toxicity triggered by anticancer drugs suppress the immune system and increase susceptibility to microbial infections[8]. Besides, many of the currently employed anticancer drugs have been incriminated with side effects and other complications on health system. For example, the drug doxorubicin has been proved to cause apoptosis of normal healthy tissues of brain, liver, kidney and also life threatening cardiotoxicity[7]. In addition, diminishing effects of anticancer drugs on account of development of drug resistance by human cells have also been reported[9].

As the antineoplastic drugs have been proved to possess the lowest therapeutic indices of any drug and cause predictable multi system toxicity[10], there is an immense need for a safe agent which can selectively kill tumor cells and enable the treatment successful. Researchers in recent years advocate the application of nanoparticles as suitable alternatives to commonly used chemotherapeutic agents. As part of scientific advancement in medicine, nanoparticles ranging in size between 0.1 and 100 nm, owing to their distinctive biological properties, are finding extensive applications in health care industry in recent years[11]. Since the discovery of cisplatin as a most effective anticancer agent way back in 1929, there is a growing attention on employing metal-based drugs as chemotherapeutic agents as antimicrobial, anti-inflammatory, antirheumatic and antineoplastic drugs[12].

With the advent of cancer nanotechnology, attempts are on the way to diagnose, prevent and treat various types of cancers including leukemia, cancers of bone, prostate, bladder, gastric and colorectal systems, etc.[13,14]. For example, Gold nanoparticles (AuNPs) have been employed for early diagnosis of chronic myeloid leukemia (CML) through molecular recognition and quantification of mRNA[15] and also for detection of circulating tumor cells (CTCs) using surface-enhanced Raman scattering (SERS) process[16]. Metal nanoparticles (MNPs) were initially used in nanomedicine as a part of drug delivery systems (nanocarriers). Owing to their much smaller size (10^{-9} m), MNPs could overcome the blood-brain barrier (BBB) and gain access into the diseased human cell and target the changes taking place at the molecular level thereby enabling the control of disease at its onset[17]. Application of many MNPs in the delivery of anticancer drugs including delivery of daunorubicin using ferric oxide[18], delivery of doxorubicin using titanium dioxide[19], etc. have been attempted successfully.

Research studies conducted in recent years have demonstrated the anticancer potential of MNPs. Inhibition of different cancer cell lines by silver nanoparticles[20-22], gold nanoparticles[23,24], Zinc oxide nanoparticles[14], titanium oxide nanoparticles[25], etc. have been well documented. Review of available literatures infer that studies employing MNPs stabilized with organic/inorganic substances for anticancer activity are scarce. The augmenting potential of polymeric stabilizers on the biological properties of MNPs have been addressed by many studies[26,27]. Therefore in the present study, it is aimed to assess the cytotoxic and anticancer properties of silver, copper and nickel nanoparticles stabilized by Polyvinyl Pyrrolidone (PVP).

2. MATERIALS AND METHODS

2.1 Synthesis, purification and characterization of Metal Nanoparticles (MNPs)

Synthesis of three MNPs (copper, nickel and silver) was carried out using a chemical method namely, polyol process[28] with minor modifications. For the purpose of achieving the stabilization of nanoparticles a polymer, polyvinyl pyrrolidone (PVP), was used during the synthesis. The powder samples of MNPs thus synthesized viz., copper (PVP-CuNP), nickel (PVP-NiNP) and silver (PVP-AgNP) were stored in an air free contained purged with N_2 gas until further use.

Subsequent to visual observation of color change in the solution, the synthesized MNPs were further subjected to characterization using powder X-ray diffraction study using X'pert PROPAN analytical instrument. The crystalline nature of MNPs were determined using Debye-Scherer's formula[29]. In order to record the surface morphology and size distribution of MNPs the transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out at an accelerating voltage of 5kV and magnification of x10k for copper and silver nanoparticles.

2.2 Assay of Cytotoxicity and Anticancer activity:

2.2.1 Cell lines used for study

Two types of cell lines viz., normal Vero cell lines (monkey kidney cells) and cancerous A549 cell lines (Adenocarcinomic human alveolar basal epithelial cells) were procured from National Center for Cell Science, Pune, India. The cells were passaged at regular intervals using Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), amphotericin B 20µg and streptomycin (1mg/ml). Maintenance of cell lines was carried out in CO_2 incubator at 37°C with 5% CO_2. Periodically the stability and purity of cell cultures were ascertained by ensuring the maintenance of confluency and absence of bacterial and fungal contamination.

2.2.2 Determination of cytotoxicity of MNPs

The property of cytotoxicity of all the three MNPs was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay[30]. For the assay 200 µL cell culture (1×10^5 cells/mL) was seeded in each wells of a 96-well micro-titration plate and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. Different concentrations viz., 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/mL of drug (PVP capped MNPs) were prepared using 1% dimethyl sulfoxide (DMSO) and each concentration of the drug was added to the cell culture (200 µL/well). A control group of untreated cells was maintained separately. This set up was incubated further for 48 h. Then to each of the well 20 µL of MTT solution (5 mg/mL), prepared in phosphate buffered saline, was added and incubated for 4 h. One mL of DMSO was added to each well so as to solubilize the formazan crystals and optical density was recorded at 540 nm using a microplate reader (Model Flx800, BioTek). The viability of cell culture in terms of relative growth rate (RGR) (%) was determined using the following formula:

$$RGR\ (\%) = A_{540\ nm} \text{ of test cells} / A_{540\ nm} \text{ of control cells} \times 100\%$$

Cytotoxic effect of the drug was calculated by graphical method and expressed in terms of IC_{50} value i.e., minimum concentration of the drug required to cause inhibition of 50% of viable cells.

2.2.3 Determination of anticancer efficacy of MNPs

The anticancer property of MNPs and its longevity was determined by toxicity grade assay prescribed by Cao *et al*.[31] with slight modifications. Culture of lung cancer cells (A549) was prepared (1×10^5 cells/mL) and seeded in four rows of the wells of microtitration plate (200 µL/well) and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. To the wells of first three rows IC_{50} concentration of MNPs (PVP-CuNP / PVP-NiNP / PVP-AgNP – prepared in 1% DMSO), thus determined through MTT assay, was added. The fourth row of cells was treated with no drug and served as negative control. The plate was incubated at 37°C and incubation of selected wells was terminated at fixed time intervals i.e., 1, 3, 5 and 7 days. At the end of each incubation corresponding wells were treated with 20 µL of MTT solution (5 mg/mL) for 4 h. Finally, 1 mL of DMSO was added to each well and the absorbance was recorded at 540 nm using microplate reader. The RGR (%) of the cell culture was determined as mentioned previously. The toxicity and longevity of the anticancer property of the drug was graded according to the resultant RGR of cells into five levels viz., grade 0: RGR $\geq$ 100%; grade 1: 100% > RGR $\geq$ 75%; grade 2: 75% > RGR $\geq$ 50%; grade 3: 50% > RGR $\geq$ 25%; grade 4: 25% > RGR $\geq$ 0%. Drugs qualifying the grades 3 or 4 were considered efficacious.

3. RESULTS

3.1 Synthesis and characterization of MNPs:

Synthesis of metal nanoparticles viz., PVP-CuNP, PVP-NiNP and PVP-AgNP was initially determined by visual inspection of corresponding color change in the reaction mix prepared with each of them. The X-ray diffractogram indicated the crystalline nature (in terms of stability, precipitation and aggregation) and the sizes of these nanoparticles as 22$\pm$1 nm (PVP-CuNP), 24$\pm$1 nm (PVP-NiNP) and 55$\pm$1 nm (PVP-AgNP) (fig 1). The spherical nature of copper and silver nanoparticles was revealed through TEM and SEM micrographs. While the copper nanoparticles appeared to be discrete, the silver nanoparticles appeared as denser and aggregated flocs and only few of them were scattered (fig 2).

3.2 Cytotoxicity of MNPs

The results of assay of cytotoxicity of MNPs on normal Vero cell line and lung cancer A549 cell line are presented in Tables 1 and 2 respectively. Subsequent to the test on Vero cell line it was observed that the MNPs of the present study exert cytotoxic effect at relatively higher concentrations. While the PVP-CuNP showed the least cytotoxicity (IC_{50} = 11.80 µg/mL) moderate effects were caused by PVP-NiNP and PVP-AgNP with respective IC_{50} values of 6.70 and 6.35 µg/mL (Table 1). Overall, the cytotoxic activity of MNPs under study on Vero cells was in the order of PVP-AgNP > PVP-NiNP > PVP-CuNP.

The cytotoxicity assay on cell line of cancerous lung cells (A549) indicated that all of the MNPs of present study could inhibit the growth of cells even at minimal concentrations. In variation to the case with Vero cell line, the least cytotoxicity on A549 cells was recorded with PVP-NiNP (IC_{50} = 1.90 µg/mL) followed by PVP-CuNP (IC_{50} = 1.75 µg/mL) and PVP-AgNP (IC_{50} = 1.40 µg/mL) (Table 2; Fig. 3). There was only marginal differences in cytotoxic activities among the MNPs tested in the study. Cytotoxic potential of these MNPs were in the order PVP-AgNP > PVP-CuNP > PVP-NiNP.

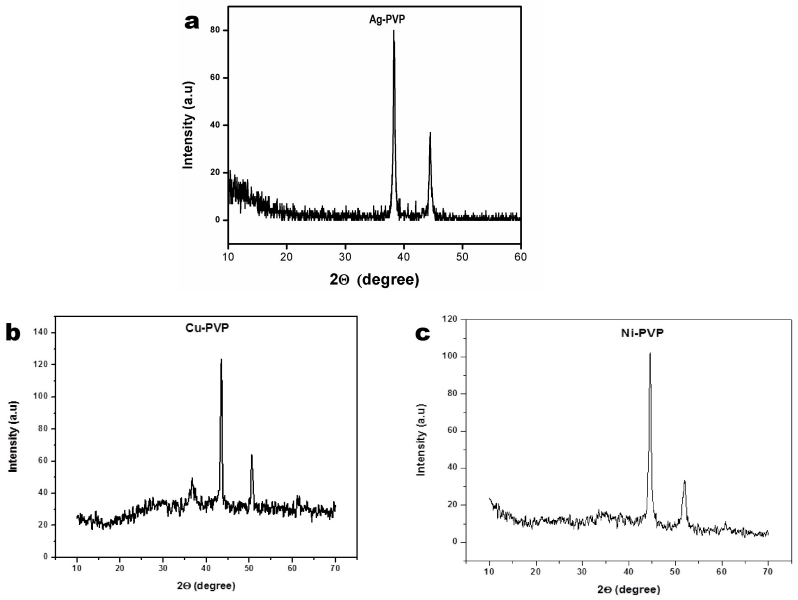

Fig. 1: X-ray diffractogram of a) AgNPs b) CuNPs c) NiNPs

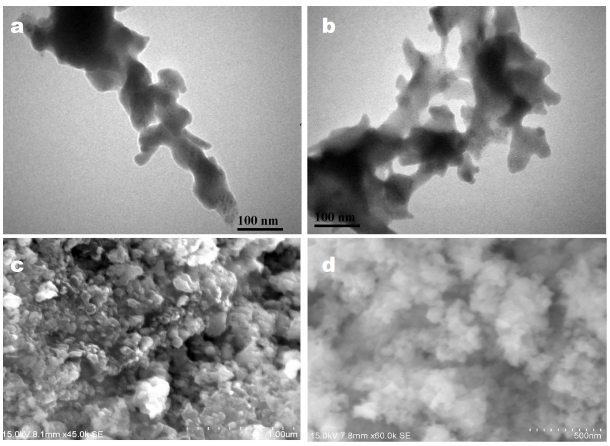

Fig. 2: TEM (a & b) and SEM (c & d) images of CuNPs and AgNPs

Table 1. Results of assay of cytotoxicity of MNPs on Vero cell line

Concentration of Drug (MNPs) (µg/mL)	Inhibitory effect (Cytotoxicity) of MNPs on Vero cell line					
	PVP-CuNP		PVP-NiNP		PVP-AgNP	
	$A_{540\ nm}$	RGR (%)	$A_{540\ nm}$	RGR (%)	$A_{540\ nm}$	RGR (%)
200	0.05	4.5	0.00	0.0	0.01	0.9
100	0.13	11.8	0.04	3.6	0.06	5.4
50	0.21	19.0	0.17	15.4	0.13	11.8
25	0.36	32.7	0.29	26.3	0.27	24.5
12.5	0.51	46.3	0.38	34.5	0.46	41.8
6.25	0.67	60.9	0.56	50.9	0.59	53.6
3.12	0.81	73.6	0.69	62.7	0.71	64.5
Control cells	1.10	100	1.10	100	1.10	100

Table 2. Results of assay of cytotoxicity of MNPs on A549 cell line

Concentration of Drug (MNPs) (µg/mL)	Inhibitory effect (Cytotoxicity) of MNPs on A549 cell line					
	PVP-CuNP		PVP-NiNP		PVP-AgNP	
	$A_{540\ nm}$	RGR (%)	$A_{540\ nm}$	RGR (%)	$A_{540\ nm}$	RGR (%)
200	0.00	0.0	0.00	0.0	0.00	0.0
100	0.00	0.0	0.00	0.0	0.00	0.0
50	0.03	2.8	0.00	0.0	0.00	0.0
25	0.05	4.8	0.03	2.8	0.00	0.0
12.5	0.11	10.5	0.09	8.6	0.05	4.8
6.25	0.15	14.4	0.19	18.2	0.17	16.3
3.12	0.29	27.8	0.34	32.6	0. 21	20.1
1.56	0.61	58.6	0.67	64.4	0.51	49.0
Control cells	1.04	100	1.04	100	1.04	100

3.3 Efficacy of anticancer activity of MNPs

The assay on evaluation of efficacy and longevity of anticancer activities of MNPs under study yielded striking results as depicted in Table 3. Among the MNPs tested, the anticancer efficacy of PVP-NiNP was observed to be comparatively inferior as it could cause the better inhibition (grade 3) of cells only from day 5 and sustained with the same grade even on day 7. The PVP-CuNP, although produced qualifying effect from day 3 (grade 3), could deploy its best inhibitory effect (grade 4) only on day 7. In contrast to these MNPs the PVP-AgNP displayed a substantial anticancer activity within a relatively shorter time of treatment (Fig. 3). It can be noted from Table 3 that even on the first day it could cause grade 2 level of inhibitory effect followed by substantial effect on the following days. Maximum lethality (grade 4) of PVP-AgNP to an extent of 80% and 90% (approx.) of growth inhibition was recorded on days 5 and 7 respectively. Obviously, in terms of demonstrating sustained anticancer cytotoxicity, the PVP-AgNP were more efficacious followed by PVP-CuNP and PVP-NiNP (Table 3).

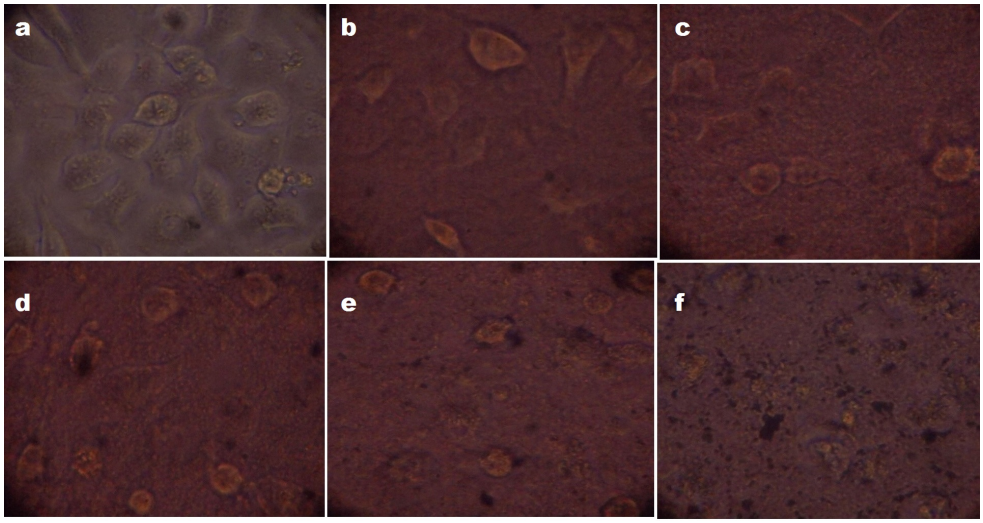

Fig. 3: Cytotoxic effect of PVP-AgNPs on Lung cancer A549 cell lines

Control (a) and cells treated with drug at concentrations of 1.56 µg/mL (b), 3.12 µg/mL (c), 6.25 µg/mL (d), 12.5 µg/mL (e), 25 µg/mL (f)

Table 3. Anticancer efficacy of MNPs

Drug (MNPs) tested	Culturing days	Anticancer activity on A549 cell line	
		RGR (%)	Toxicity Grade
PVP-CuNP	1	80.5	1
	3	39.7	3
	5	32.5	3
	7	22.7	4
PVP-NiNP	1	92.2	1
	3	54.5	2
	5	39.5	3
	7	29.3	3
PVP-AgNP	1	65.4	2
	3	31.8	3
	5	21.2	4
	7	10.3	4

4. DISCUSSION

The metal nanoparticles (MNPs), owing to their extraordinary specificity and precise action, are being explored for extensive applications in various fields of science. In order to combat the growing challenge posed by cancer disease, various metal nanoparticles endowed with pharmacological potentials have been investigated. Research studies conducted in recent years have demonstrated the anticancer properties of different MNPs including, gold nanoparticles against cancer cells of colon (Col320)[24], liver (Hep G2), lung (A549)[23], cervix (HeLa), kidney (HEL-293), colorectal adenocarcinoma (K-562) and bone marrow (K562)[32]; zinc oxide nanoparticles against cells of myeloblastic leukemia (HL60)[14]; titanium oxide (TiO_2) against hepatocarcinoma (SMMC-7721) cells[19]; ferric (Fe2O3), copper (Cu2O) and titanium oxide (TiO2) composites against human breast cancer (MCF-7) cells[25]; ruthenium(II)-arene nanoparticles against lung cancer (HCC827) cells[33]; silver nanoparticles against cells of epidermoid larynx carcinoma (Hep-2)[34] , colon adenocarcinoma (HT 29)[35], colon (MCT) and breast (MCF-7) cancer[36], liver (HepG2) and Prostate (PC3) cancer[37], etc.

The present study initially focused on demonstrating the cytotoxic properties of MNPs on normal Vero cell line and cancerous A549 lung cells. Among the MNPs experimented the PVP-CuNPs showed better efficiency i.e., it caused cytotoxicity on normal cells only at higher concentration (IC_{50} = 11.80 µg/mL) while its toxic dose for cancer cells was below 2.0 µg/mL (Tables 1 & 2). Owing to their innocuous and less toxic nature[38], in general, studies on exploring cytotoxic potential of CuNPs are scanty. Bondarenko *et al*.[39] from their review of data of literatures indicated that the median LC_{50} of CuONPs on mammalian cells was 25 mg/mL, which is much higher than the cytotoxic concentration of CuNPs of the present study. Recently Sivaraj *et al*.[40] have demonstrated moderate anticancer activity of CuONPs on MCF-7 breast cancer cell lines. The longevity of anticancer efficacy of PVP-CuNPs of the present study has been satisfactory as it could cause $\geq$ grade 3 level of inhibition of A549 cells from the day 3 of culturing onwards (Table 3). Concomitant with our finding Aazam *et al*.[41] portrayed the anticancer activity of CuNPs against cervical (HeLa) and breast cancer (MCF-7) cell lines at very low LD_{50} concentrations.

The PVP-NiNPs of this study demonstrated moderate and comparatively higher cytotoxicity on normal cells (IC_{50} = 6.70 µg/mL) and on cancer cells (IC_{50} = 1.90 µg/mL) respectively (Tables 1 & 2). However, it showed a poor anticancer efficacy as it could exhibit grade 3

level of cytotoxicity only on the day 5 of cell culture with no significant progressive activity until day 7 (Table 3). Guo *et al*.[42] explained from their study on inhibition of hepatocellular carcinoma cells (SMMC-7721) that the NiNPs exhibit particl anticancer activity at a concentration of 12.5 µg/mL and the total activity require a dose of as high as 50 µg/mL. The study by Ahmed *et al*.[43] demonstrated that in order to cause the inhibition of breast cancer (MCF-7) cells the NiNPs consumed IC_{50} concentrations of >50 µg/mL, which is much higher than that of the requirement of PVP-NiNPs of the present study. Another study deciphered complete lacking of anticancer activity of NiNPs against HeLa and MCF-7 cell lines[41]. These studies are in support of our finding that NiNPs are comparatively inferior in their anticancer potential.

Survey of literature inferred that the AgNPs are the most explored nanoparticles for anticancer potential among the different MNPs studied so far. The lethality of AgNPs is believed to occur through induction of toxic reactive oxygen species, damage of mitochondria and DNA - all leading to cell death[27]. The PVP-AgNPs tested in the present study were moderate and more efficient with respect to the cytotoxic activities on normal cells (IC_{50} = 6.35 µg/mL) and on cancer cells (IC_{50} = 1.40 µg/mL) respectively (Tables 1 & 2). The study of Renugadevi *et al*.[21] demonstrated that the AgNPs required an IC_{50} dose of 90 µg/mL for inhibiting human epidermoid larynx carcinoma (Hep-2) cell lines. Another recent study revealed the requirement of 90 µg/mL as IC_{50} dose against Hep-2 cells[44]. More recently Remya *et al*.[45] from their experiment with AgNPs found that the NPs require an IC_{50} of 7.19 µg/mL for exerting anticancer activity against breast cancer (MCF-7) cells. In contrast to these findings, the PVP-AgNPs of the present study exhibited notable anticancer activity even at minimal concentrations. The AgNPs employed in the study of Sankar *et al*.[46] consumed an IC_{50} of 100 µg/mL against human lung cancer (A549) cells and required a treatment period of 36 h. Astonishingly, the results of the present study unveiled that PVP-AgNP could display substantial anticancer activity on A549 cells with an IC_{50} dose of 1.40 µg/mL starting from day-1 of culturing with grade 2 level of action and achieving grade 4 as early as in five days (Table 3).

The chemical methods, although efficient in yielding, are frequently encountered with a flaw of producing floundering MNPs. Such MNPs tend to agglomerate resulting in reduced display of interfacial free energy and limited surface area. This could be overcome by the application of stabilizers[47]. Therefore the present study employed PVP polymers as chemical agents to bring about the stability of metal nanoparticles. The augmenting effect of PVP on the MNPs has been evident from superior anticancer activity depicted by PVP capped MNPs employed in the present study. While the PVP-NiNPs and PVP-AgNPs of this study could exhibit higher inhibitory activity even at lower concentrations, the uncapped counterparts of these MNPs deployed in other studies[21,42,43,45] could establish their lethality only at higher concentrations. In line with our finding, the study of Guo *et al*.[27] have demonstrated the anticancer activity of PVP capped AgNPs against acute myeloid leukemia (THP-1) cells with IC50 dose of as low as 0.86 µg/mL.

5. CONCLUSION

The growing prevalence of cancer around the world poses alarming challenges to ensure medical care to human kind. Metal nanoparticles (MNPs), as advocated by many research studies, could be considered as vital anticancer drugs in view of their specific and precise lethal activity. Application of polymeric substances such as PVP could enhance the biological activity of MNPs. Among the three types of MNPs experimented in the study, the PVP-CuNPs, although least cytotoxic on normal (Vero) cells, displayed short lasting efficacy on cancer (A549) cells. The PVP-NiNPs exhibited moderate and considerable cytotoxic activities respectively on normal and cancer cells. Despite showing moderate cytotoxicity on normal cells the display and longevity of anticancer potential by PVP-AgNPs has been substantial. Further studies on PVP-AgNPs, in terms of purification and exploring anticancer potential against different cancer cells, are suggested to develop it as a promising anticancer drug.

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